Zn stimulates the phospholipids (PL) biosynthesis via the pathways of oxidative and endoplasmic reticulum (ER) stress in the intestine of freshwater teleost yellow catfish

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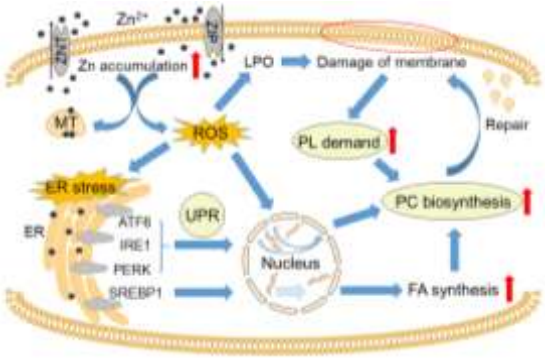
ABSTRACT:

The hypothesis of our study was that waterborne Zn exposure evoked phospholipids (PL) biosynthesis to compensate for the loss of membrane integrity, and the pathways of oxidative stress and ER stress mediated the Zn-evoked changes of PL biosynthesis. Thus, we conducted RNA sequencing RNA-seq to analyse the differences in the intestinal transcriptomes between the control and Zn-treated *P. fulvidraco*. 56-day Zn exposure increased the intestinal Zn accumulation, and mRNA levels of 816 genes were markedly up-regulated and 263 genes down-regulated. Many DEGs in pathways of PL biosynthesis and protein processing in ER were identified. Their expression profiles indicated that waterborne Zn exposure injured protein metabolism and induced PL biosynthesis, caused oxidative stress and ER stress, and activated the unfolded protein response. Then, using the primary enterocytes, we identified the mechanism of oxidative and ER stress mediating Zn-induced PL biosynthesis, and indicated that the activation of these pathways constituted adaptive mechanisms to reduce Zn toxicity. Our study demonstrated that Zn exposure via the water increased Zn accumulation and PL biosynthesis, and that oxidative stress and ER stress were interdependent and mediated the Zn-induced PL biosynthesis of the intestine in the freshwater teleost.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The pollution of aquatic ecosystems by metals is of concern due to their toxicity and bioaccumulation. Among these metals, zinc (Zn) is an essential trace element for many important biochemical processes in vertebrates including fish, but can be toxic above the threshold concentration in the body. Among aquatic organisms, fish are considered to be the ideal organism for pollution monitoring in aquatic ecosystems,[1] and unique among the vertebrates because they have two routes of metal acquisition, from the diet and from the water.[2] In contrast to marine fish, freshwater fish do not drink considerable volumes of water for osmotic homeostasis, and waterborne Zn is absorbed mainly via the gill.[3] The influence of waterborne Zn on gill has been well studied due to its direct involvement in Zn toxicity in freshwater fish.[4] The intestine is also very sensitive to a wide range of stressors.[5] In our pilot study, we found incidentally that waterborne Zn exposure increased Zn accumulation in the intestine of yellow catfish. We found this interesting and toxicologically relevant because increased Zn accumulation in the intestine could potentially influence the physiological functions of the intestine. However, whether and how waterborne Zn had effects on physiological functions of the intestine are not exactly known in freshwater teleost, and the underlying mechanisms of intestinal homeostatic regulation are not well understood.

In fish, the failure to maintain Zn homeostasis results in the damage of cellular structure and intracellular membranes, and impedes a wide range of physiological processes.[6] Therefore, it is meaningful to emphasize that Zn exposure via water can increase intestinal Zn accumulation and in turn initiate the change of intestinal physiological functions. Phospholipids (PL) are the major lipid components of cellular

and intracellular membranes.[7,8] Despite the importance of PL in maintaining integrity and physiological state of membrane, no studies have addressed the effects of mineral elements (including Zn) on PL metabolism. Since excess Zn in the water can damage cellular structures, we hypothesize that waterborne Zn can affect PL biosynthesis in the intestine.

The ER, an important organelle, is responsible for folding and assembly of proteins, PL synthesis and Ca^{2+} homeostasis. The changes in the ER increase the amounts of unfolded/misfolded proteins in the ER, evoking ER stress and increasing the concentration of Ca ion in the cytoplasm.[9] To recover the ER homeostasis, cells activate the UPR, a pathway governed by three sensors of ER stress: PERK, IRE1 and ATF6 (their full names were shown in Abbreviation list in Supporting Information, SI).[8] Together, they evoke gene transcription to increase folding capacity and down-regulate protein synthesis, and induce elimination of unfolded proteins through ER associated degradation (ERAD).[10] At present, increasing evidence indicates that the UPR is involved in the regulation of lipid and PL biogenesis.[11,12] However, the molecular mechanism linking the UPR to PL biogenesis has not been elucidated. In addition to ER stress, Zn also induces oxidative stress[13,14] by accelerating the generation of ROS.[15] Moreover, generation of ROS during oxidative stress directly or indirectly (or both) affects ER homeostasis and UPR activation.[16,17] Therefore, we reasoned that this ROS-based model would be very helpful in illuminating the undefined mechanism linking ER stress to Zn-induced changes of PL metabolism.

Here, we hypothesize that Zn exposure via the water affected PL metabolism, and that the pathways of oxidative stress and ER stress mediated the Zn-induced alterations of PL metabolism. Therefore, using RNA-seq technology, the present study ascertained the effects of waterborne Zn influencing physiological responses of the intestine in *Pelteobagrus fulvidraco*. Using primary yellow catfish enterocytes, the complex relationship between Zn and oxidative stress/UPR/PL metabolism was investigated. Yellow catfish, an omnivorous freshwater teleost, is widely cultured in several Asian countries and used as experimental animals. We reveal an unprecedented role for the ER stress and oxidative stress mediating Zn-induced changes of PL biosynthesis.

2. MATERIALS AND METHODS

The study consisted of three experiments. All experiments followed the ethical guidelines of Huazhong Agricultural University.

2.1. Expt. 1: Experimental procedures and sample analysis

2.1.1. Zn exposure and sampling

The experimental procedures for waterborne Zn were described in our parallel study.[9] Briefly, 144 healthy yellow catfish (mean body weight: 8.4 ± 0.4 g) were were randomly transferred to 6 fiberglass tanks (300 L water, 24 fish per tank). They were exposed to two nominal Zn concentrations: 0 (control, no extra Zn addition) and 7.69 μM (0.5 mg/L, 5% of the 96-h LC_{50}[18]), respectively, with triplicate tanks for each treatment. Zn was added in the form of $ZnSO_4$ $7H_2O$. Here, the Zn concentnration (0.5 mg/L) was lower than those in some contaminated waters in China, and also lower than these in water quality criteria II according to GB 3838-2002 of China (1.0 mg Zn/l, GB3838-2002). All fish were fed twice daily with diets (Zn content: 17.45 mg/kg diet). The actural Zn concentrations for two treatments were 0.007 ± 0.001 and 0.514 ± 0.070 mg/l, respectively.[19] The experiment continued for 56 days, and the entire intestine samples (including anterior, mid- and posterior intestine) were collected for the determination of contents of triglyceride (TG) and metal elements, enzymatic activity and mRNA expression.

2.1.2. Measurements of metal, TG content and enzymatic activities

The intestine Zn content was measured using ICP-AES.[19] Quality assurance/quality control (QA/QC) procedures included analysis of three method blanks (purified water), three certified reference tissues (DORM-2, LUTS-1 and DOLT-2) and two randomly selected duplicate samples.

Activities of G6PD, FAS, 6PGD, ME and ICDH (their full names were listed in SI) were analyzed based on the methods described in our previous study.[19] The TG content and activities of Cu, Zn-SOD were measured by using commercial kits (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China).

2.2. Transcriptome analysis

2.2.1. RNA isolation, library preparation and sequencing

Three fish per tank were randomly selected for RNA isolation. Trizol reagent (Invitrogen, CA, USA) was used for total RNA isolation. In total, three fish from each tank and three replicates per treatment (six samples in total) were used for

transcriptomic sequencing. The construction of cDNA library and sequencing (Illumina HiSeq 2000 platform, Illumina, CA, USA) were undertaken at the Beijing Genome Institute (BGI, Beijing, China) according to the methods described previously.[19]

2.2.2. De novo assembly and annotation

Before assembly, clean reads were obtained from raw data by filtering adaptor sequences and low-quality sequences. They were then *de novo* assembled into unigenes using Trinity.[20] We annotated the transcripts over 200 bp using the BLASTx alignment (e-value < 10^{-5}) between unigenes and databases, including NR (non-redundant protein sequence), non-redundant nucleotide (Nt), Swiss-Prot, KEGG (Kyoto Encyclopedia of Genes and Genomes) and COG (Clusters of Orthologous Groups). Blast2GO was used to accomplish Gene Ontology (GO) annotation ,[21] and WEGO program for further gene classification .[22]

2.2.3. Differentially expressed genes (DEGs) and Q-PCR validation

After obtaining *de novo* transcriptomic data, the BGISEQ-500 platform (BGI, Beijing, China) was used to quantify the expression levels of genes. 6 independent cDNA libraries (three equivalent quantities of RNA samples from the same tank were combined into one pool, and there were three replicate tanks and accordingly 3 cDNA libraries per treatment) were constructed for RNA-Seq quantification analysis (all reads have been submitted to the Sequence Read Archive at NCBI, Accession Number: SRP116984). The gene expression levels were calculated using the fragments per kilobase of transcript per million mapped reads (FPKM) method[23] and DEGs were screened using Noiseq method[24] with a value of $\log_2$fold-change > 1 and a diverge probability ≥ 0.8. All DEGs were mapped to terms in GO and the KEGG database. In the present study, twelve candidate genes were randomly selected for Q-PCR validation. [19] Primers are given in Table S1 (SI). A set of seven housekeeping genes (*β-actin*, *b2m*, *elfa*, *gapdh*, *rpl7*, *hprt* and *tbp*) were selected from the literature[25] in order to test their transcription stability. The mRNA levels of *β-actin* and *hprt* were the most stable, based on the analysis of geNorm software[25] Accordingly, the gene expression levels were normalized to the geometric mean of the *β-actin* and *hprt*. The $2^{-\Delta\Delta Ct}$ method[26] was used to calculate the fold changes in relative expression to the control.

2.3. Expt. 2: *in vitro* studies

2.3.1. Enterocytes culture and treatment

Enterocytes were isolated from yellow catfish, based on the published methods[27,28]. Four pathway inhibitors were used, and they are TPEN (Zn^{2+} chelator, Sigma, MO, USA), NAC (ROS inhibitor, Selleck, TX, USA), fatostatin (SREBP1 inhibitor, Selleck, TX, USA) and STF-083010 (IRE1 inhibitor, Selleck, TX, USA). Ten groups were designed as follows: control, 40 μM Zn, 2 μM TPEN, 1mM NAC, 10 μM fatostatin, 50 μM STF-083010, 40 μM Zn + 2 μM TPEN, 40 μM Zn + 1 mM NAC, 40 μM Zn + 10 μM fatostatin and 40 μM Zn + 50 μM STF-083010. The concentrations of Zn and inhibitors were selected according to our pilot trials and to other *in vitro* studies.[9,29-31] Each treatment was performed in triplicates. The cells were collected at 48 h for following analysis.

2.3.2. Cell viability, TG and PC content

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.[19] Intracellular TG content was measured as described above. The amount of phosphatidylcholine (PC) was measured using a PC assay kit (Sigma, MO, USA).

2.3.3. Zn^{2+} and Ca^{2+} measurements

Two cell permeant probes, Newport Green DCF (Ex/Em = 505nm/535nm) and Fluo-4 (Ex/Em = 494nm/516nm) were used to measure intracellular Zn^{2+} and Ca^{2+} concentrations, respectively[9,32] Fluorescence was detected and quantified using laser scanning confocal microscopy (Leica, Wetzlar, German).

2.3.4. Flow cytometry for the determination of apoptosis and ROS

Apoptosis analysis was conducted with 5 μl Annexin V-FITC and 5μl propidium iodide (PI), accompanied with flow cytometry (Beckman, CA, USA) with ModFit 3.0 LTTM software.

ROS production was measured with 10 μM 2, 7-dichlorofluorescin diacetate (DCFH-DA).[33] Fluorescence intensity was measured at the wavelengths of excitation at 490 nm and emission wavelength at 525 nm, respectively.[34] For fluorescence intensity measurement of MCLA (2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-α] pyrazin-3-one), a specific chemiluminescence probe for superoxide anion and singlet oxygen, the PBS-washed cells were incubated with 8 μM MCLA for 20 min. Fluorescence intensity was

measured at the wavelengths of excitation at 430 nm and emission wavelength at 546 nm, respectively.

2.3.5. Enzymatic activity, MDA content and Q-PCR

Activities of G6PD, FAS, 6PGD, ME and ICDH were analyzed following the methods in our previous study.[19] The malondialdehyde (MDA) content and activities of total-SOD and Cu, Zn-SOD were measured by commercial kits (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China). Soluble protein content of the homogenates was determined by the method of Bradford.[35]

Q-PCR for the *in vitro* experiment was performed based on the methods mentioned above, and*β-actin* and *tbp* were used as the control genes according to geNorm software.

2.4. Expt. 3: Bioaccumulation of Zn in tissues

Considering that dietary Zn might have the potential influence on the Zn uptake, we further performed an experiment to analyze the relative contribution of dietary Zn and waterborne Zn. 180 yellow catfish (initial mean weight: 10.8 ± 0.3 g) were randomly stocked in 12 tanks and there were 15 fish per tank. The yellow catfish were exposed to four treatments for 8 weeks, three replicates for each treatment. There were two waterborne Zn concentrations: 0 (no extra Zn addition in the water) and 7.69 μM (0.5 mg/L, waterborne Zn addition group) waterborne Zn. For each waterborne Zn exposure, fish were fed 6.17 (dietary Zn deficiency group) or 17.45 mg/kg (dietary Zn addition group) Zn, respectively. The group of dietary Zn deficiency, TPEN (Zn chelator) was added at 0.01 g/kg to inhibit dietary Zn uptake. The feed composition and nutrient analysis of diets were shown in Table S6 (SI). The fish were fed diets at a ration of 4% of body weight twice a day. Fish were sampled on days 0, 14, 28, 42 and 56, respectively. Gills, intestine and plasma were sampled for the determination of Zn content and mRNA expression of genes involved in Zn uptake and transport.

2.5. Statistical analysis

Statistical analysis was conducted with SPSS 19.0 software.[19] Data were analyzed with one-way ANOVA and Student's t-test where appropriate. Significant level was set at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Waterborne Zn had direct effects on Zn accumulation and PL metabolism of the intestine

In the freshwater fish, Zn is assimilated both from waterborne and dietary phases. In the present study, Zn exposure via the water significantly increased Zn accumulation (Fig. 1A). 1079 unigenes were identified as DEGs by RNA-seq technology, including 816 up- and 263 down-regulated genes (Fig. S1, SI). The GO (Fig. S2, SI) and KEGG (Fig. S3, SI) database were further used to perform the pathway analysis (For detailed RNA-seq data and validation result, see SI, Table S2-S5, Fig. S4). The results indicated that waterborne Zn exposure impacted the physiological function of the intestine in many aspects and a series of genes and pathways may be of importance for Zn-induced physiological responses. Similar to the results in the *in vivo* study, intracellular Zn content of primary enterocytes were up-regulated after Zn incubation, and the changes were partly reversed by TPEN (Fig. S5, SI), which to some extent supports the argument. Overall, the present study indicated that waterborne Zn disrupted the Zn balance in the intestine, which thereby led to diverse physiological changes, similar to our study in yellow catfish hepatocytes.[33] The present study found an interesting contradiction: Zn increased activities of enzymes involved in fatty acid (FA) synthesis (Fig. 1D) but reduced TG content (Fig. 1B) in the intestine. Considering that FA is not only incorporated into TG, but also for the synthesis of phosphatidic acid (PA), PL precursor, we accordingly analyzed the DEGs involved in PL biosynthesis pathway. The present study found that many genes, such as *acc*, *fas*, *acsl*, *gpat*, *agpat*, *cds*, *ck*, *pap*, *ept1* and *cept1*, involved in FA and PL biosynthesis, were up-regulated by waterborne Zn exposure (Fig. 2). The activities of enzymes related to FA synthesis, such as *6pgd*, *me*, *icdh* and *fas*, were significantly increased by waterborne Zn exposure (Fig. 1D). These observations indicated that waterborne Zn exposure induced PL biosynthesis. Similarly, several studies pointed out that the synthesis of PL was regulated in response to Zn status in yeast.[36,37] Iwanyshyn et al.[36] found that the activities of the CDP-diacylglycerol pathway enzymes were positively associated with the cytoplasmic Zn levels. GPAT and AGPAT were key enzymes for the initiation of PL synthesis.[7] PC is the main PL and the precursor to SM, PS and PE.[12] Some key enzymes: CK, CCT, CHPT1/CEPT1 and PEMT, played important roles in PC biosynthesis.[12,38-40] Considering that waterborne Zn increased tissue MDA content (Fig. 1C) and PC is the main membrane PL in cells,[12] it is plausible to speculate that

increased PC synthesis compensated for the loss of membrane integrity induced by Zn exposure. Moreover, our study found that the expression of DGAT, a crucial enzyme catalyzing formation of TG from DG, was markedly decreased. Thus, it is reasonable to conclude that most of FA participated in the PL biosynthesis process, not in TG biosynthesis. To verify whether increased Zn content directly affected these changes in the intestine, Zn^{2+} chelator TPEN was used in the *in vitro* study. Our study indicated that TPEN significantly attenuated the Zn-induced increase of expression of *accα*, *gpat*, *pap*, *cds*, *cct* and *ect* (Fig. 3A), which in turn suppressed the Zn-induced accumulation of PC content and the reduction of TG content (Fig. 3B), as observed here.

3.2. Waterborne Zn evoked ER stress and UPR

Zn exposure influenced the protein metabolism and processing in ER (Fig. 4). For instance, the expression levels of *uggt* and *bip* were markedly elevated. UGGT and BiP are parts of the ER quality control system and preferentially recognizes the unfolded/misfolded proteins.[10] Thus, the increased expression of *uggt* and *bip* probably reflected the increase of these unfolded/misfolded proteins in the ER. The current study also showed that mRNA levels of *perk*, *atf6* and *ire1* were significantly up-regulated (Fig. 4). It has been reported that under ER stress, aberrant proteins rapidly accumulates and accordingly induces the UPR in eukaryotic cells.[10] Hence, these events suggested that waterborne Zn caused the UPR in the intestine of yellow catfish. One of the adaptive mechanisms that recover normal physiological state of ER is the upregulation of the folding capacity through induction of molecular chaperones.[8] Here we found that expressions of chaperones, such as *bip* and *cnx*, were up-regulated, indicating the increased folding capacity in response to Zn exposure. Moreover, Zn increased the expression of many genes from ERAD and ubiquitin proteolysis, including the glycosylase *ermani* which catalyzes the important step in ERAD.[41] The enhanced ERAD was conducive to degrade and remove excess unfolded/misfolded proteins in ER. It has been postulated that activation of UPR increases ERAD efficiency through the IRE1-XBP1 cascade.[8,10] As expected, expression levels of *ire1* and *xbp1* were up-regulated here, which constituted a pivotal link between Zn-induced UPR and ERAD. Similarly, Sriburi et al.[42,43] also reported that XBP-1 stimulated the expression of many genes in fibroblasts, including those encoding the protein translational machinery.

3.3. The activation of IRE1-XBP1 pathway was involved in Zn-induced PL biosynthesis

Many enzymes involved in PL biosynthesis, such as GPAT and AGPAT, are related with the ER.[7,44] Studies also suggested that PL biosynthesis was induced in a response to ER stress in the yeast.[45] Thus, we further explored whether Zn-induced occurrence of ER stress and UPR mediated PL biosynthesis. Here, we found that Zn exposure increased the expression of genes in ER stress and UPR, such as *perk*, *atf6*, *ire1*, *bip* and *cnx* (Fig. 3A), indicating their activation. TPEN markedly inhibited the Zn-induced up-regulation of mRNA abundance of *grp94*, *crt*, *ire1α* and *perk*, further indicating that Zn induced the occurrence of ER stress and UPR *in vivo* and *in vitro*. Similarly, van der Sanden et al.[46] suggested that the UPR might provide a means to rapidly respond to an increased need for PL in Chinese hamster ovary cells. The present study indicated that STF-083010 (IRE1 inhibitor) significantly suppressed the Zn-induced up-regulation of mRNA expression of *ire1α*, *xbp1*, *cds*, *cct* and *cept* (Fig. 5A). However, STF-083010 had no suppressed effect on Zn-induced increase of Zn content (Fig. S6, SI) and TG content (Fig. 5B). These findings suggested that CDS, CCT and CEPT, the key regulatory factors involved in the PC biosynthesis, were potential targets of IRE1-XBP1 ptahway, similar to the result by Sriburi et al.[42] XBP1 is also closely linked with PC synthesis.[10] Enforced expression of XBP1 is sufficient to induce PC biosynthesis.[42] Other studies indicated that the level and synthesis of CCTa protein, was up-regulated in XBP-1(S)-transduced fibroblasts, and accordingly elevated production of cytidine diphosphocholine (CDP-choline).[43] The activation of ER stress and UPR by waterborne Zn exposure has been reported in the yellow catfish liver tissues.[9] Other reports pointed out that during a physiological UPR, the IRE1-XBP1 pathway regulated synthesis of PL according to cellular needs for ER membrane components and its activation of IRE1/XBP1 branch of the UPR responded to increased demand for PL in B cells and fibroblasts.[12,42] Thus, the XBP1 was activated, presumably to facilitate PL biosynthesis under the ER stress,[42,43] which was essential for optimal cellular functions in response to Zn stimulation. In addition, unmitigated ER stress led to apoptosis.[10,47,48] Similarly, the present study indicated that inhibition of IRE1 pathway during Zn incubation had a negetive effect on apoptosis (Fig. S7, SI) and cell viability (Fig. S8, SI). Thus, inhibition of IRE1 hindered the remedy process of UPR, which likely injured the cells and initiated the apoptosis to eliminate unhealthy cells, as suggested by Sriburi et al.[43]. Therefore, all

these observations above indicated that the UPR, especially the IRE1-XBP1 pathway, played a positive role in alleviating ER stress and maintaining normal functions of ER, at least partly via facilitating PC biosynthesis.

3.4. SREBP1 mediated the regulation of Zn-induced PL biosynthesis

SREBP1 is a transcription factor that regulate multiple genes involved in FA synthesis, including *acc* and *fas*.[49] The present *in vivo* experiment found that Zn increased the *srebp1* mRNA levels. Thus, *srebp1* seemed to play key roles in Zn-induced alterations of PL metabolism.[49-51] Using the SREBP1 inhibitor fatostatin (F), we found that, compared to the control, the PC content in the Zn-treated group was increased while the PC content in the Zn + F group was relatively stable (Fig. 6C), indicating that *srebp1* was involved in Zn-induced PC biosynthesis. Compared to the control, activities of ME, G6PD and FAS, and mRNA levels of *srebp1*, *accα*, *fas* and all the tested genes involved in PC synthesis and PC content were enhanced by Zn incubation (Fig. 6A and 6B), further confirming the results of the transcriptome analysis. ACCα, G6PD and FAS are the crucial enzymes during FA biosynthesis.[52] SREBP1 inhibitor caused the reversion of Zn-induced increase of *fas* and *accα* mRNA expression (Fig. 6A), and increase of G6PD and FAS activities (Fig. 6B), thereby possibly impacting fatty acid synthesis. For genes involved in PL synthesis, compared to single Zn treatment, fatostatin pre-treatment suppressed the changes of *gpat*, *pap* and *cds*. Given the close correlation between the synthetic pathways of fatty acid and PC, SREBP1 plays important roles in PC biosynthesis and SREBP1 activation may be required for providing enough fatty acid to support PC biosynthesis.

3.5. The Zn-induced changes of physiological functions was partially attributable to oxidative stress

To investigate how Zn exposure induced ER stress, we explored the change of Ca^{2+} concentration in the enterocytes. However, we did not observe appreciable Ca^{2+} release (Fluo-4) after Zn addition (Fig. S9, SI). Considering that intracellular free radicals, such as ROS, directly or indirectly affect ER homeostasis, and thereby lead to UPR.[16,17] We further determine the parameters involved in ROS generation. Here we detected the increased fluorescence intensity of ROS (Fig. S10, SI), increased activities of T-SOD and Cu, Zn-SOD, increased MDA content and increased MCLA fluorescence intensity in the Zn-treated group (Fig. 7C), suggesting the accumulation of intracellular ROS. Cu, Zn-SOD is one of the key free-radical scavengers.[53] The

upregulated Cu, Zn-SOD activity probably implied the production of cellular superoxide anion radicals in yellow catfish intestines in a response to Zn exposure. Similarly, previous studies also demonstrated that Zn stimulated the production of ROS.[14,54] In contrast, Zn incubation seemed to have no effect on ROS production in yellow catfish hepatocytes.[33] ROS augments ER stress.[55,56] Our study indicated that increasing Zn led to the generation of ROS and oxidative stress, and preincubation with NAC prevented the Zn-evoked ROS generation, indicating that oxidative stress was responsible for the Zn toxicity, in agreement with other studies.[54] As a result, the increased expression levels of *bip*, *crt*, *ire1α* and *perk* in the Zn-treated group was partly reversed in the Zn + N group (Fig. 7A), indicating that Zn-induced ER stress was, at least in part, attributable to generation of ROS. Meantime, NAC attenuated the Zn-induced effects on PC content (Fig. 7B) and expression of genes involved in PL biosynthesis (Fig. 7A), suggesting the involvement of ROS generation in Zn-induced PL biosynthesis. High concentration of ROS can inflict direct damage to proteins and lipids.[16,57] In this study, Zn treatment significantly increased intracellular MDA content. MDA has been widely used as a biomarker for lipid peroxidation (LPO).[57] Taken together, Zn-induced ROS accumulation probably caused the peroxidation, which further impaired proteins, lipids and membrane integrity. Other research also suggests that Zn caused lipid peroxidation and loss of membrane.[58,59] The demand for membrane PL components would increase correspondingly and thereby caused a series of adaptive mechanisms, including the activation of IRE1-XBP1 and SREBP1 pathways which initiated a cascade of biochemical events that enhanced supplies of PL and alleviated stress. Thus, the present study provided the first evidence that Zn-induced ROS and UPR signaling mutally mediated Zn-induced PL biosynthesis in enterocytes.

3.6. Zn accumulation in tissues was dominated by waterborne uptake

Fish can uptake Zn from diet and from gill-plasma route. A potential problem is that dietary Zn might have the potential influence on the intestinal Zn accumulation, which might further influence the results. In order to clarify the effect, we analyzed the effects of dietary and waterborne Zn exposure on Zn deposition (Fig. S11, SI). No surprisingly, waterborne Zn exposure resulted in significant Zn accumulation in the gill on day 56, similar to the study in rainbow trout.[60] However, dietary Zn had no significant effects on gill Zn accumulation. Plasma Zn concentration ranged from 10 to 25 μg/ml and increased significantly with time in waterborne Zn groups. Other

studies also reported that waterborne Zn exposure increased Zn concentrations in the plasma.[61-63] Plasma Zn concentrations are maintained within narrow margins in the fish[61] and alterations may mean the occurrence of toxicity.[63] Thus, it seemed that Zn exposure via the water evoked the perturbations of plasma Zn and toxicity, whereas the dietary exposure did not. Sappal et al.[63] also found that chronic dietary Zn exposure had no significant effect on plasma Zn content in rainbow trout. Similar to the plasma, Zn exposure via the water increased the intestine Zn content much more than dietary exposure did (Fig. S11 A, SI). Previous study in yellow catfish indicated that only an extremely high dietary Zn concentration (213.84 mg/kg) had nearly the same effect on tissues Zn accumulation as 0.35 mg/L waterborne Zn.[64] In view of the fact that dietary Zn content we used was much lower than 213.84 mg/kg and belongs to the normal demand range for yellow catfish (dietary Zn requirement for yellow catfish: 17.1-20.9 mg Zn/kg diet)[65], our study found that the intestine Zn accumulation came mainly from gill-plasma route. Furthermore, changes of dietary Zn concentration did not result in changes of mRNA levels of *znt1* and *zip4* on day 56, whereas waterborne Zn exposure increased *znt1* expression and decreased *zip4* expression compared to the group without Zn addition in the water (Fig. S12, SI), indicating that waterborne Zn, but not dietary Zn, influenced mRNA expressions of genes involved in Zn uptake and transport. Taken together, under the present condition, our study strongly indicated that Zn accumulation and changes in the physiological functions (PL biosynthesis) in the intestine were predominantly influenced by waterborne Zn exposure. Our results are in agreement with the opinion that Zn via the water is toxicologically more significant probably because Zn permeates the gill more easily.[63]

In conclusion, for the first time, our data provide evidenced that Zn induced PL biosynthesis by activating the pathways of oxidative stress and ER stress. Our study offered new insights for elucidating the effects and mechanism of Zn toxicology, which will be potential meaningful for incoming Zn risk assessments in aquatic environment.

ASSOCIATED CONTENT

Supporting information

Table S1, primers used for Q-PCR analysis; Table S2, statistics of reads and assembly quality; Table S3, summary of annotation and CDS results; Table S4, summary of

sequencing data for each sample; Table S5, alignment statistics of reads align to reference gene; Table S6, feed formulation and proximate analysis of experimental diets ; Figures S1, the correlation between the gene expression profiles of control and Zn-exposed groups; Figures S2, GO functional classification of DEGs; Figures S3, KEGG functional classification of DEGs; Figures S4, comparison of mRNA levels between RNA-seq and Q-PCR results; Figures S5, effect of Zn and/or TPEN on intracellular Zn fluorescence; Figures S6, Effect of Zn and/or inhibitors on intracellular Zn fluorescence; Figures S7, effect of Zn and/or STF-083010 (IRE1 inhibitor) on apoptosis; Figure S8, Effect of Zn and/or inhibitors on cell viability; Figures S9, intracellular Ca fluorescence after Zn stimulation; Figures S10, effect of Zn and/or NAC on intracellular ROS generation; Figure S11, effect of waterborne and dietary Zn on tissues Zn accumulation; Figure S12, Effect of waterborne and dietary Zn on mRNA levels.

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Notes

The authors declare no competing financial interest.

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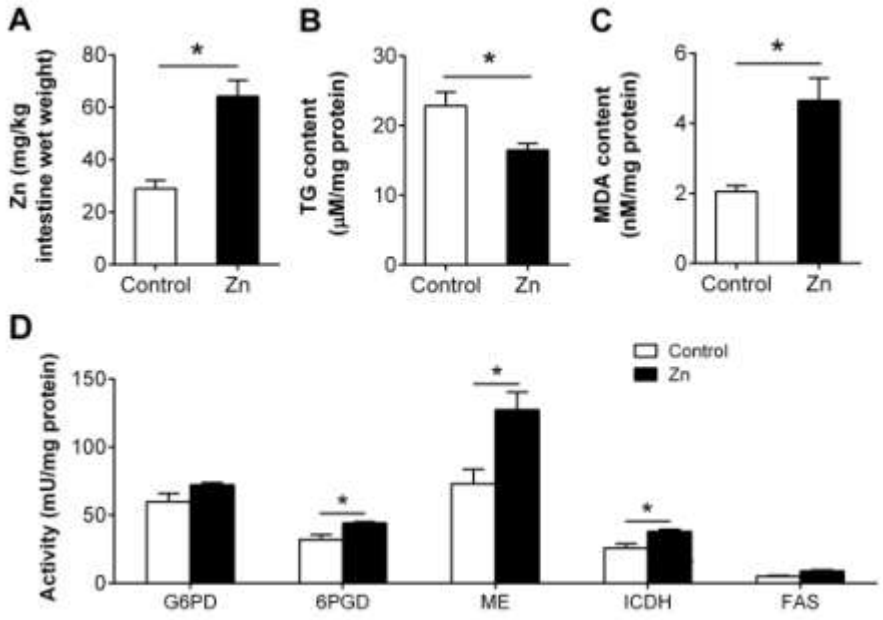

Fig. 1 Effect of waterborne Zn exposure on Zn (A), TG contents (B), MDA content (C) and enzymatic activities (D) in the intestine of *P. fulvidraco* on day 56. Values are means ± SEM (n= 3 replicate tanks, six fish were sampled for each tank). Asterisks (*) indicate significant differences between control and Zn-exposed group.

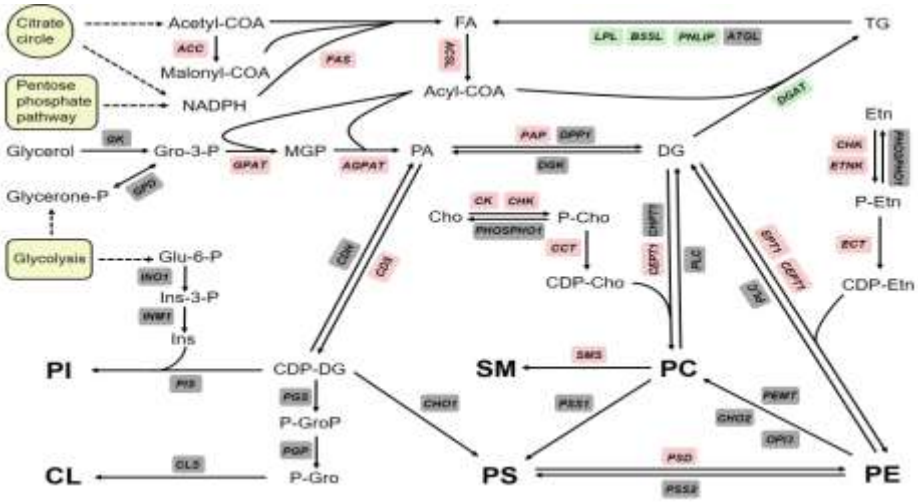

Fig. 2 Differentially expressed genes involved in phospholipid biosynthesis. The up-regulated genes (Probability ≥ 0.8, and the absolute value of $\log_2$ (Ratio) ≥ 1) are highlighted in red. The down-regulated genes (Probability ≥ 0.8, and the absolute value of $\log_2$ (Ratio) ≥ 1) are highlighted in green. The non DEGs are highlighted in grey. FA, fatty acid; PA, phosphatidic acid; DG, diacylglycerol; CL, cardiolipin; SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; Etn, ethanolamine. The abbreviations of genes are shown in the Abbreviation List of the Supplementary file.

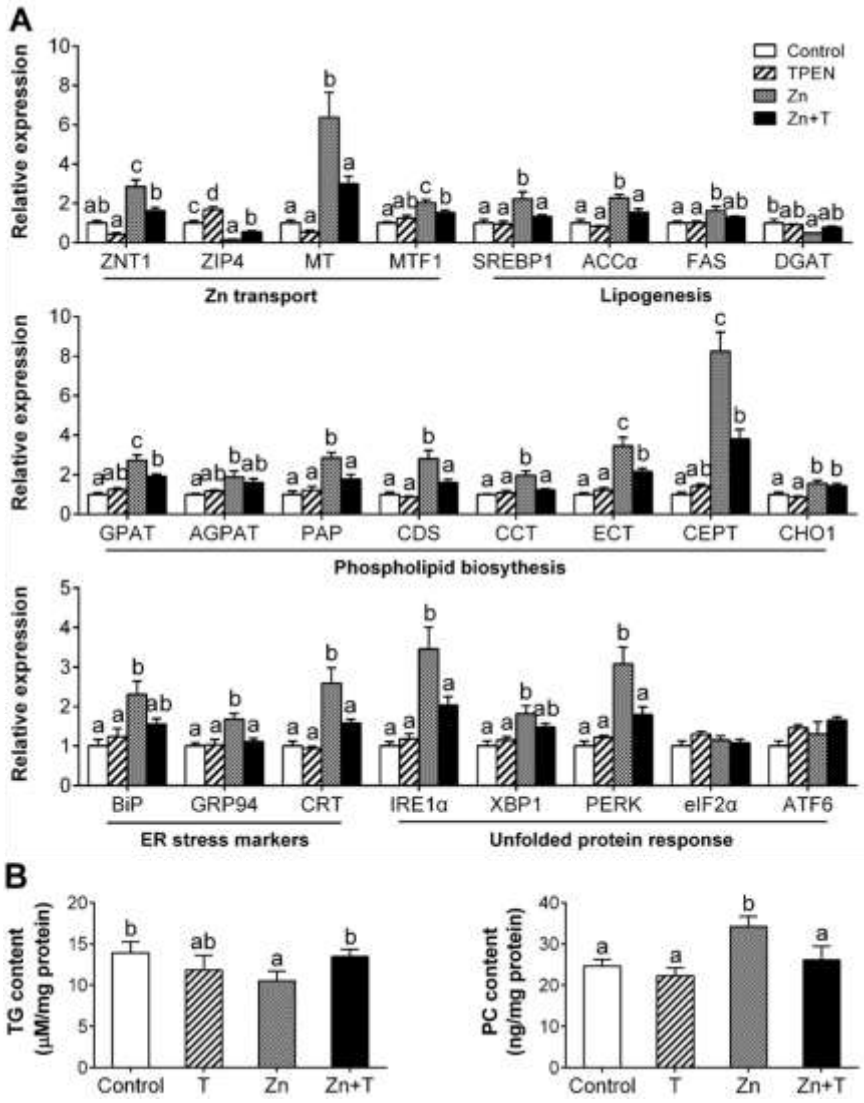

Fig. 3 Effects of Zn and/or TPEN (Zn^{2+} chelator) on mRNA levels (A), TG accumulation (B) and PC content (C) in enterocytes of yellow catfish at 48 h. Values are means ± SEM (n = 3). Bars that share different lowercase letters indicate significant differences among groups ($p < 0.05$).

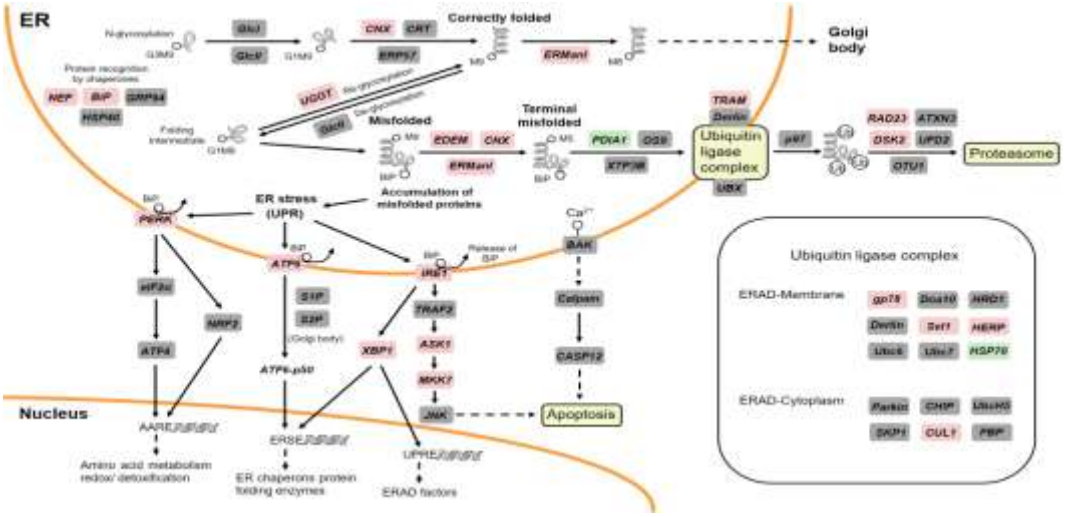

Fig. 4 Differentially expressed genes involved in protein processing in ER. The up-regulated genes (Probability ≥ 0.8, and the absolute value of $\log_2$ (Ratio) ≥ 1) are highlighted in red. The down-regulated genes (Probability ≥ 0.8, and the absolute value of $\log_2$ (Ratio) ≥ 1) are highlighted in green. The non DEGs are highlighted in grey. G3M9: (Glc)3 (GlcNAc)2 (Man)9 (Asn)1; G1M9: (Glc)1 (GlcNAc)2 (Man)9 (Asn)1; M9: (GlcNAc)2 (Man)9 (Asn)1; M8: (GlcNAc)2 (Man)8 (Asn)1; M5, (GlcNAc)2 (Man)5 (Asn)1; Ub, ubiquitin. The abbreviations of genes are shown in the Abbreviation List of the Supplementary file.

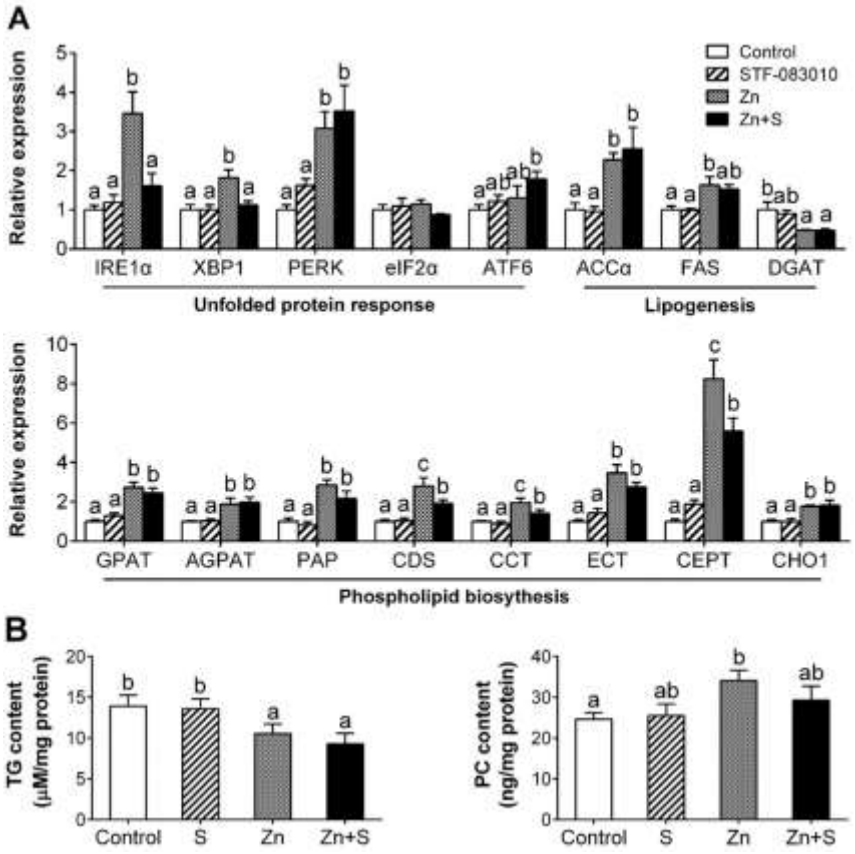

Fig. 5 Effects of Zn and/or STF-083010 (IRE1 inhibitor) on mRNA levels of genes involved in UPR, lipogenesis and PL biosynthesis (A), TG accumulation and PC content (B) in enterocytes of yellow catfish at 48 h. Values are means ± SEM (n = 3). Bars that share different lowercase letters indicate significant differences among groups ($p < 0.05$).

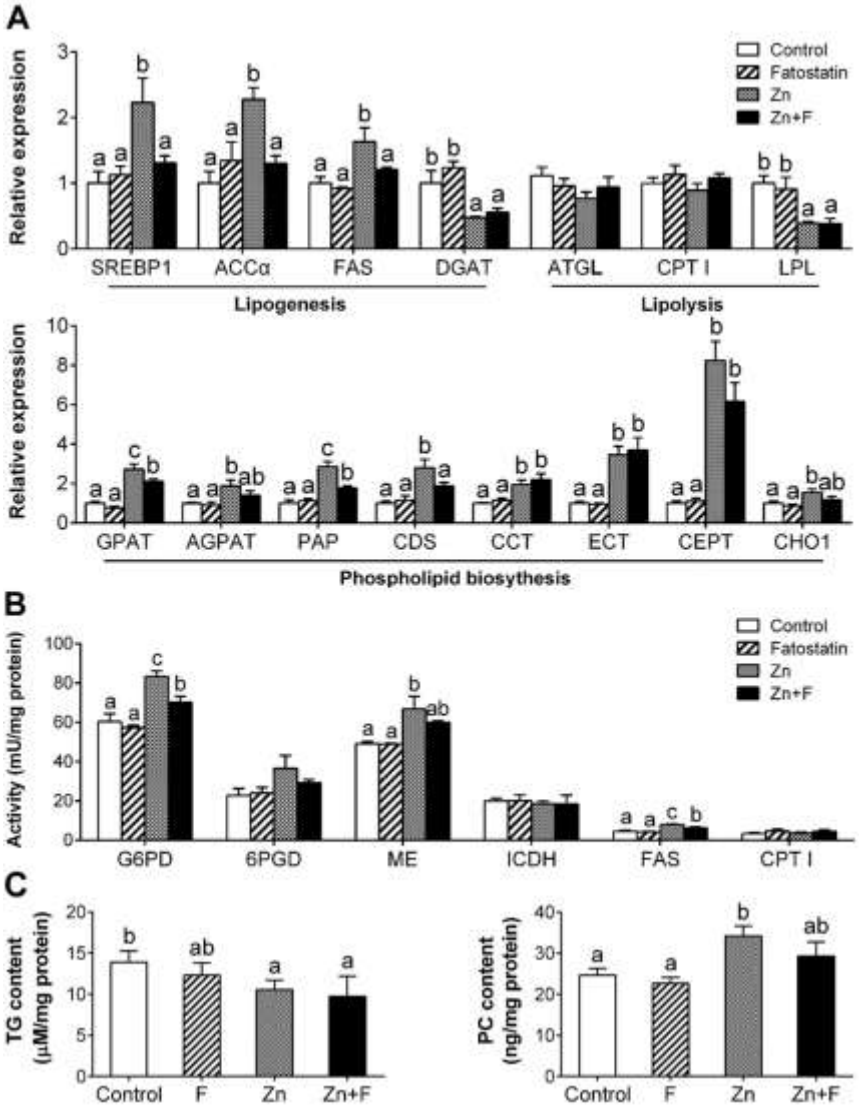

Fig. 6 Effects of Zn and/or fatostatin (SREBP1 inhibitor) on mRNA levels of genes involved in lipogenesis, lipolysis and PL biosynthesis (A), enzymatic activities (B), TG accumulation and PC content (C) in enterocytes of yellow catfish. Values are means ± SEM (n = 3). Bars that share different lowercase letters indicate significant differences among groups ($p < 0.05$).

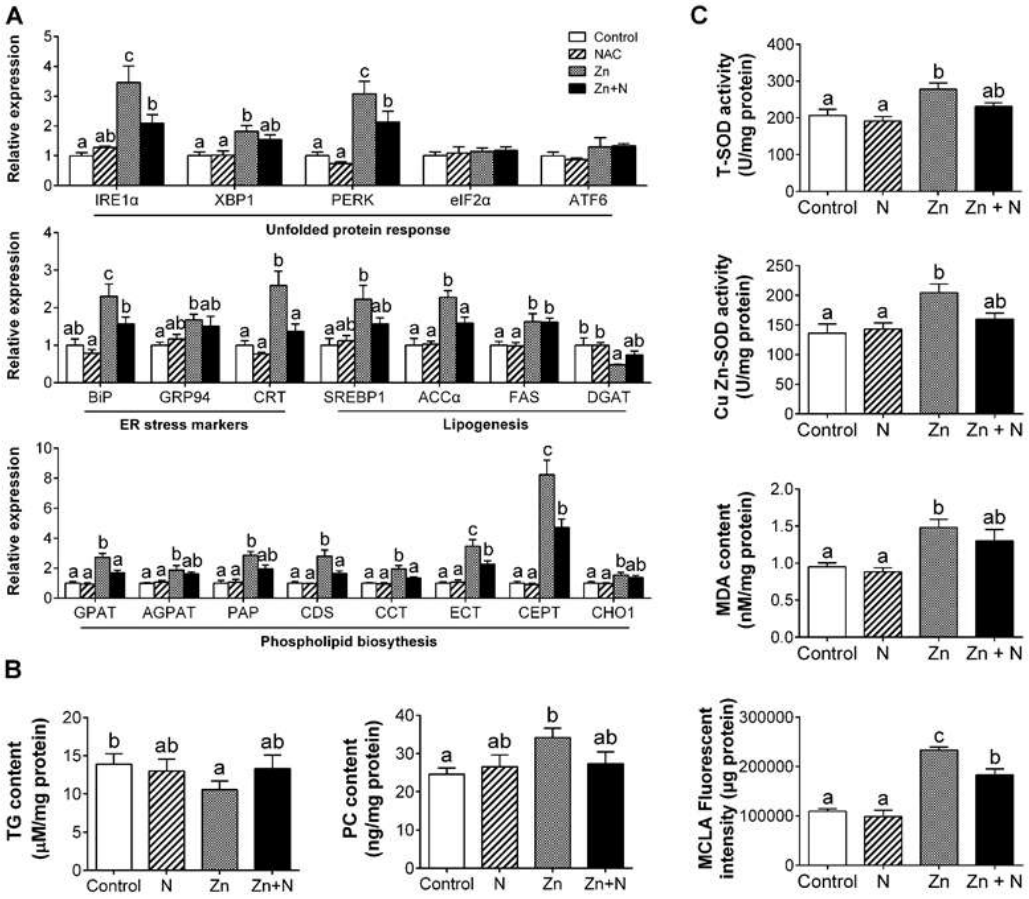

Fig. 7 Effects of Zn and/or NAC (ROS inhibitor) on mRNA levels of genes involved in UPR, ER stress markers, lipogenesis and PL biosynthesis (A), TG accumulation and PC content (B), SOD activities, MDA content and MCLA fluorescent intensity (C) in enterocytes of yellow catfish. Values are means ± SEM (n = 3). Bars that share different lowercase letters indicate significant differences among groups ($p < 0.05$).